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ORIGINAL ARTICLE

Novel CXCR4 Inhibitor CPZ1344 Inhibits the Proliferation, Migration and Angiogenesis of Glioblastoma

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Abstract

Glioblastoma (GBM) are life-threatening tumors with a poor prognosis and low cure rates. GBMs are malignant brain tumors that develop from astrocytes. Most GBMs are not inherited and occur sporadically. GBM recurrence after standard treatment has led to the assessment of agents targeting the CXCR4 chemokine receptor as alternative drug target for much needed GBM therapeutics. In present study, a novel CXCR4 inhibitor modified with a picolinamide scaffold (CPZ1344) was designed and synthesized. Its anti-GBM function was then evaluated. Our results showed that CPZ1344 reduced the growth of GBM cells in a concentration dependent manner. The anti-GBM activity of CPZ1344 was due to alteration in GBM-cell morphology and apoptotic induction in GBM cells. CPZ1344 inhibited the migration and angiogenesis of U87 cells, led to cell cycle arrest in the G1 phase and inhibited CXCR4 signaling. These findings demonstrate the anticancer effects of CPZ1344 and its potential as a novel anti-GBM therapeutic.

Keywords CXCR4 · CPZ1344 · Migration · Glioblastoma

Abbreviations

GBM	glioblastoma multiforme
HUVECs	Human umbilical vein endothelial cells
TUNEL	Terminal deoxynucleotidyl transferase-mediated Dutpnick end-labeling

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Introduction

Malignant brain tumors are leading causes of cancer related death [1]. Glioblastoma (GBM) is a highly prevalent genetic central nervous system malignancy occurred in both adults and children with low cure rate. Median time to progression of GBM patients after standard treatments is 7 months and median overall survival is only 14 months [2]. Enhanced proliferation rates, increased drug resistance and migration ability promote GBM development and progression, tumorigenic cancer stem cells also contribute to GBM initiation and therapeutic resistance [3]. Despite the technological advancements in surgical techniques, radiotherapy, and chemotherapy regimens, the average survival time of GBM patients remains low. When combined with radiotherapy, the alkylating agent Temozolomide can improve overall survival (OS) in GMB patients [4]. However, tumor recurrence is inevitable after initial therapy [5]. New therapeutic targets are therefore urgently required to improve GBN associated OS and to prevent tumor recurrence.

The recurrence of GBM is closely associated with its increased invasive and migrate ability [6]. The invasive phenotype of GBM can be regulated by several mechanisms, and recent studies implied that chemokine receptor CXCR4 regulates tumor invasiveness, recurrence, and metastasis [7].

CXCR4 is overexpressed in neuronal and astrocyte brain tumors, with enhanced expression associated with poor patient prognosis [8]. CXCL12, a CXCR4 ligand, is similarly overexpressed in GBM cells and cancerous blood vessels [9]. The CXCL12-CXCR4 axis regulates the proliferation of GBM cells in the CNS [10]. These highlights both CXCL12 and CXCR4 as potential therapeutic targets for much-needed anti-GBM therapies.

Small molecule inhibitors of CXCR4 have been designed that show promise as novel anti-cancer agents though their ability to inhibit cancer related metastatic phenotypes both in cell culture systems and in in vivo animal models [11]. In this regard, the systemic administration of the small-molecule CXCR4 inhibitor AMD3100 impairs the growth and invasiveness of GBM cells [12]. AMD3100 is the first reported bicyclam-containing small molecule CXCR4 antagonist that has been approved by the FDA for stem cell mobilization [13]. The efficacy of AMD3100 with bevacizumab in high-grade GBMs I sunder assessment in current clinical trials (NCT01339039). However, the sustained administration of AMD3100 leads to a range of side effects, including cardiotoxity due to the effects of the compound on macrocyclic structures [14]. In addition, AMD3100 lacks of oral bioavailability due to its polyamino structural feature. Development of novel CXCR4 antagonist to overcome these side effects of AMD3100 became an attractive yet challenging work.

Herein, we report the development of a novel, small molecule CXCR4 inhibitor, CPZ1344, and report it effects on the proliferation and migration of GBM (Fig. 1). CZP1344 was designed from WZ811 [15], another potent CXCR4 inhibitor, based on the overlapping structural features of AMD3100 through the replacement of key N-macrocyclic regions that mediate CXCR4 binding. The results of this study demonstrate that targeting CXCR4 using CPZ1344 decreases the proliferation, migration and angiogenesis of GBM.

Materials and Methods

Drugs and Cell Culture

U87, SHG44, and U251 cells (human GBM) were culture in RPMI 1640 containing 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin. Healthy human astrocytes and human umbilical vein endothelial cells (HUVECs) were grown in F12K medium plus 10% FBS and pen/strep. Cells were cultured at 37 °C at 5% CO_2. CPZ1344 was synthesized in-house by one step conduction reaction from 1,4-diaminobenzene and 2-picolinic acid in almost quantitative yield. ^{1}H-NMR (300 M HZ, DMSO-d_6) δ 9.86 (2H, s), 8.77 (2H), 8.37 (2H), 8.02 (2H), 7.93 (2H), 7.64 (4H, d, J = 7.8 Hz); HRMS calcd for $C_{18}H_{14}N_4O_2$ 318.1117, found 319.1192 $[M + H]^+$; purity 98%. The study was approved by the ethics committee of Affiliated Kunshan Hospital of Jiangsu University, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

MTT Assay

MTT assays were employed to assess the growth and viability of U87 and SHG44 cells following CPZ1344 treatment. Briefly, 5×10^4 cells were seeded into 96-well plates at 37 °C for 24 h and cells were treated with CPZ1344 at a range of concentrations for 72 h. MTT reagent was then added and absorbance's at 560 nm were read on a plate reader. Values were normalized to untreated controls.

Cell Migration Analysis

Transwell (Corning, NY, 14831, USA) was used to assay the cell migration in vitro. Treated cells were plated in the upper chamber in serum-free medium. Then, 20% FBS was added to the medium in the lower chamber. After incubating for 24 h, non-migrating cells were removed from the top well, the bottom cells were stained with 0.1% crystal violet for 30 min at 37 and then washed with PBS for twice. Then, migrating cells were photographed in three independent fields for each well. And this trial was repeated for three times.

Cell Apoptosis Assay by Flow Cytometry

For apoptosis assays, 5×10^4 cells were seeded into 6-well plates for 24 h and treated with a range of concentrations of CPZ1344 for 36 h. Cells were then washed in PBS, fixed in 4% PFA and cell nuclei were Hoechst stained (20 μg/mL) for 30 min. Cells were imaged on a fluorescent microscope and nuclear fragmentation or blebbing in response to CPZ1344 were assessed.

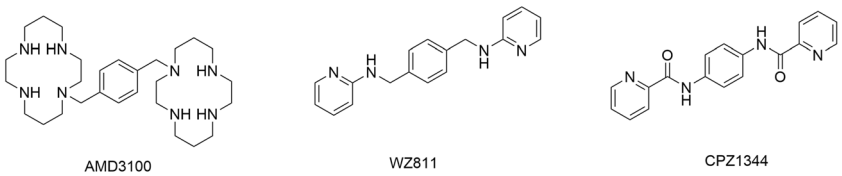

Fig. 1 Structures of the CXCR4 inhibitors AMD3100, WZ811 and the newly developed CXCR4 inhibitor CPZ1344

Cell Cycle Analysis

For cell cycle analysis, cells (5×10^4 cells/well) were seeded into 6 well plates and treated with the indicated concentrations of CPZ1344 for 36 h. Cells were detached, fixed in 70% ethanol in PBS (-20 °C) overnight and re-suspended in PBS supplemented with 100 μg/mL RNase and 50 μg/mL PI for 30 min. Cell cycle analysis was performed on a flow cytometry (FC500, Beckman Coulter).

TUNEL Staining

To assess nuclear DNA fragmentation, TUNEL assays were performed (Terminal deoxynucleotidyl transferase-mediated Dutpnick end-labeling) using commercially available kits (KGA7025, Beyotime, China). Cells seeded onto coverslips were washed with PBS and fixed in 3.7% formaldehyde for 10 min. Cells were then treated with 10% H_2O_2 followed by TdT reagent for 1 h to enable end-labeling. Cells were then washed and treated with 3',3-diamino benzidine for 10 min and counter-stained with methyl green. The % of apoptotic nuclei (TUNEL-positive cells) were counted on a fluorescent microscope (n = 100 cells per treatment quantified).

Western Blotting

Cells (5×10^5 cells/mL) in 6 well plates were treated with 80 μM CPZ1344 and lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein content in the lysates were determined by Bradford's assay and 50 μg were separated by SDS-PAGE electrophoresis. Proteins were transferred to membranes and probed with human anti-CXCR4 and anti-β actin antibodies as a loading control. Membranes were then labelled with the appropriate HRP conjugated IgG and protein bands were visualized using ECL. The density of radiographic band onto PVDF membranes was analyzed by using the software of Quantity One (Bio-Rad, Hercules, CA, USA).

Cell Morphology and Angiogenesis Assessments

HUVECs (50,000 cells/well) were seeded into 6 well plates containing pre-thawed polymerized Matrigel. Cells were treated with a range of CPZ1344 concentrations for 6 h at 37 °C and cell morphology and tube formation (as an indicator of angiogenesis) were imaged on an inverted light microscope (OLYMPUS, Japan).

Statistical Analysis

All data are presented as the mean ± SD. The SPSS Graduate Pack 11.0 was used for all data comparisons. Significant differences between CPZ1344 treatment groups were compared using a one-way ANOVA (*, $P < 0.05$, **, $P < 0.01$, and *** $P < 0.001$ significance level).

Results

CXCR4 is Upregulated in GBM

CXCR4 is known to be overexpressed in an array of human brain tumors. To reveal the role of CXCR4 in GBM, we first determined its expression in a range of human GBM cell lines. As shown in Fig. 2a, we found that the expression of CXCR4 mRNA levels was much higher in U87 SHG44 and U251 cells, compared with human normal astrocytes. These findings were further confirmed through western blot analysis that identified elevated CXCR4 protein in GBM cell lines (Fig. 2b).

CPZ1344 Inhibited the Metastatic Profiles of GBM Cells

To investigate the activity of CPZ1344 on the metastatic characteristics of GBM cells, the viability of U87 after treatment with various concentrations of CZP1344 was analyzed by MTT assays. CPZ1344 (5, 10, 20, 40, and 80 μM) inhibits the growth of U87 cells in a dose dependent manner after treatment for 72 h (Fig. 3a, left). The percent of live cells after treatment with 80 μM CPZ1344 for 72 h is only 27.6%. CZP1344 also inhibited the growth of SHG44 cells effectively, but this cell line was slightly less sensitive to CZP1344 than U87 cell line (Fig. 3a, right). The percent of live SHG44 cells after treatment with 80 μM CPZ1344 for 72 h is 31.4%. In addition, we also analyzed the migration activity of U87 cells after treatment with 5 μM CPZ1344, at which concentration, it does not affect the growth of U87 cells. The mean number of cells migrating into the lower chambers significantly declined in response to CPZ1344 treatment (Fig. 3b). The ligand CXCL12 is expressed in tumor-associated blood vessels and/or tumor cells, suggesting a paracrine relationship for CXCR4 activation. In order to evaluate whether CPZ1344 blocks the in vitro effects of CXCL12 on GBM growth, CXCL12-induced growth of U87 cells was tested by adding 5 μM CPZ1344. Interesting, CPZ1344 blocked CXCL12-induced proliferation in U87 cells (Fig. 3c), suggesting the specific binding of CPZ1344 to CXCR4.

CPZ1344 Induced Cell Apoptosis

We investigated whether apoptosis was involved in the CPZ1344 treated U87 cells. Nuclear fragmentation and/or blebbing were assessed via Hoechst 33258 staining (Fig.

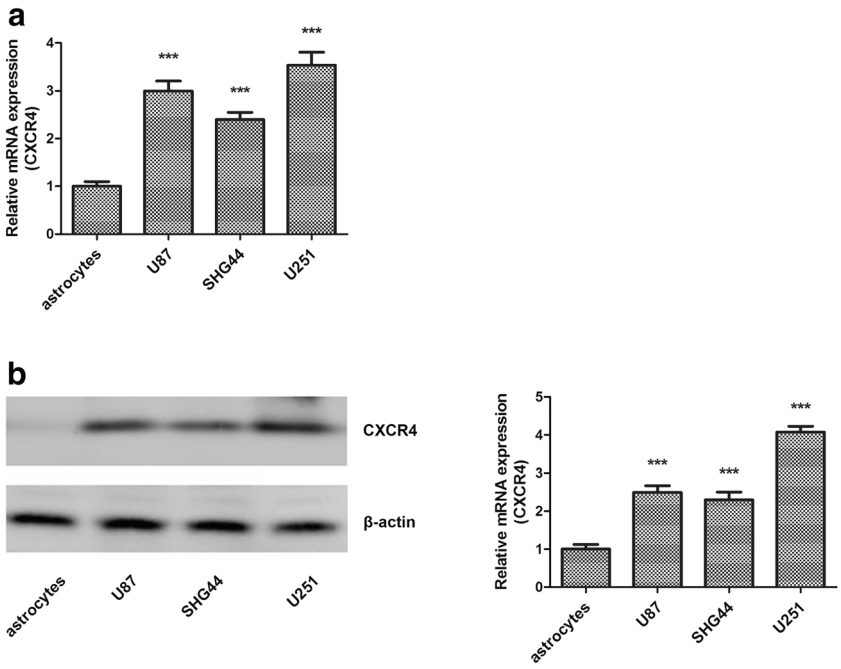

Fig. 2 CXCR4 is upregulated in GBM. **a** RT-PCR assessment of CXCR4 mRNA levels in GBM cells. **b** Western blot of CXCR4 expression in the GBM cell lines. Data are the mean ± SD (n = 03). *** P < 0.001

4a). U87 cells treated with 20, 40, and 80 μM CPZ1344 for 36 h displayed chromatin condensation and dramatic changes in cell morphology, which indicated apoptosis. Whereas in control group, treated with DMSO, no condensed chromatin was observed. The quantification of cells in later apoptosis stages were further assessed via TUNEL staining. Treatment with CPZ1344 induced apoptosis with apoptotic rates increasing from 2.1% (control) to 36.2% in the presence of 80 μM CPZ1344 (Fig. 4b). These data highlighted CPZ1344 as a potent inducer of apoptosis in U87 cells that likely explain its anti-GBM activity.

CPZ1344 Treated GBM Cells Show G1 Arrest

We next investigated the effects of CPZ1344 on GBM cell cycle progression, a key mediator of cancer cell survival and tumorigenesis. In the experiments that followed, GBM cells were treated with a range of concentrations of CPZ1344 and its effects on cell cycle status were measured via flow cytometry (Fig. 5a). We found that the number of cells in G2/M phase decreased significantly after treatment with CPZ1344, which was accompanied by an increase in cells in the G1/0 phase.

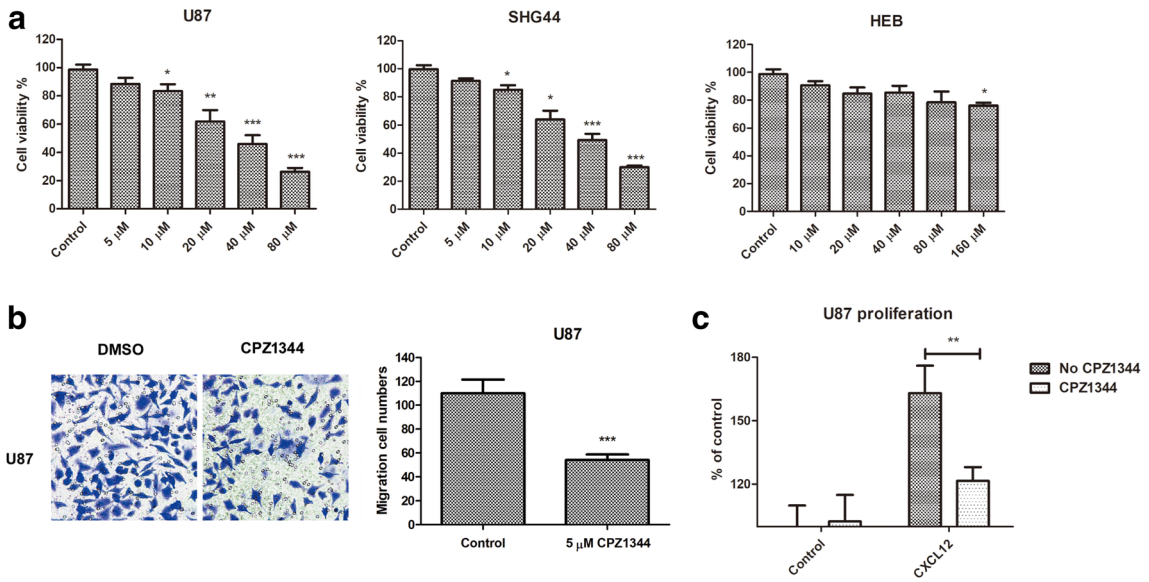

Fig. 3 CPZ1344 prevents the growth of aggressive GBM cells. **a** U87, SHG44 and HEB cells treated with CPZ1344 for 72 h. MTT assays were used to determine cell growth (mean ± SD, n = 03). *P < 0.05, **P < 0.01, ***P < 0.001. **b** CPZ1344 treatment prevented U87 cell migration. ***P < 0.001 compared to untreated controls. **c** Proliferation of U87 cells in response to CXCL12 is blocked by CPZ1344. **, P < 0.01

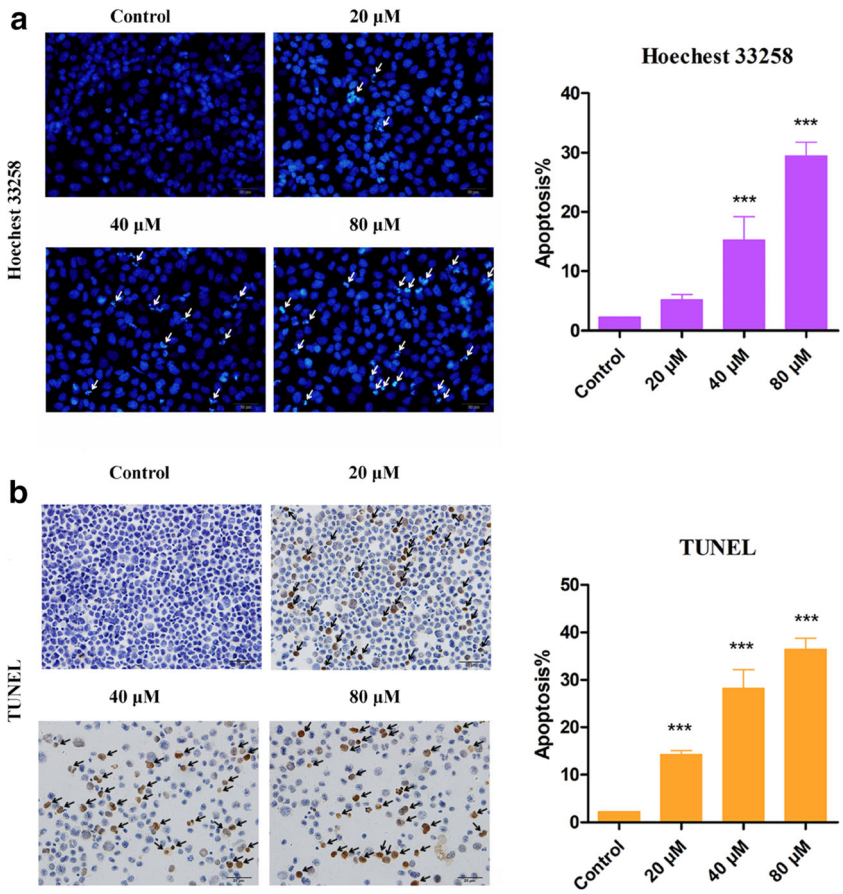

Fig. 4 CPZ1344 induces apoptosis in U87 cells. **a** Nuclear fragmentation and blebbing in CPZ1344 treated U87 cells was assessed by Hoechst 33258 staining. Cells were imaged on a fluorescent microscope. **b** DNA fragmentation as a marker of the late stages of apoptosis was assessed by TUNEL assays in response to CPZ1344 treatment. Arrows represent positive cells. The % of apoptotic cells in each treatment group as shown. Data are the mean ± SD (n = 03). ***p < 0.001 vs. untreated controls

Upon assessment of the expression of key cell cycle markers by western blot analysis (Fig. 5b) CPZ1344 treatment for 36 h led to enhanced p53 and p21 expression, with a concomitant loss of CDK2 expression. Taken together, these data suggest that CPZ1344 mediates its effects on GBM cell proliferation and survival through influencing the p53/p21/CDKs axis to promote G1 arrest.

CPZ1344 Influences CXCR4 Expression and Signaling

The results obtained to this point suggest that the antiproliferative activity of CPZ1344 in GBM cells was closely related to its effects on apoptosis. To confirm these findings, we next investigated the effects of CPZ1344 on the expression of known and well characterized apoptotic proteins including Bax, Bcl-2, and caspase-3. Bax and cleaved caspase-3 expressed increased after CPZ1344 treatment for 36 h, while the expression of Bcl-2 significantly declined (Fig. 6a). These data suggested that the intrinsic apoptotic pathway regulates CPZ1344-mediated GBM cell apoptosis. To further understand the mechanism(s) of CPZ1344 on cell proliferation in vitro, the effects of CXCR4 on known proliferative cell signalling pathways were assessed by western blot analysis. U87 cells in CPZ1344-treated group exhibited low level of CXCR4, and the activation of p-AKT and p-mTOR are also decreased compared to the control group (Fig. 6b).

CPZ1344 Exhibited Potent In Vitro Anti-Vascular Activity

The formation of tube-like vascular structures in a critical stage during the angiogenesis required for tumor formations[16]. We therefore used HUVECs to investigate the effects of CPZ1344 on vascular activity and tube formation. HUVECs in the control group formed capillary-like tubules with clear multicentric junctions, whilst treatment with CPZ1344 significantly inhibited the formation of these HUVEC tubular cords (Fig. 7). To exclude the possibility that the effects of CPZ1344 were mediated by cytotoxic activity, the IC50 of CPZ1344 against HUVECs after 6 h-treatment was detected. The result showed that CPZ1344 inhibited the growth of HUVECs with an IC50 value of 365.1 ± 45.3 μM, indicating that the

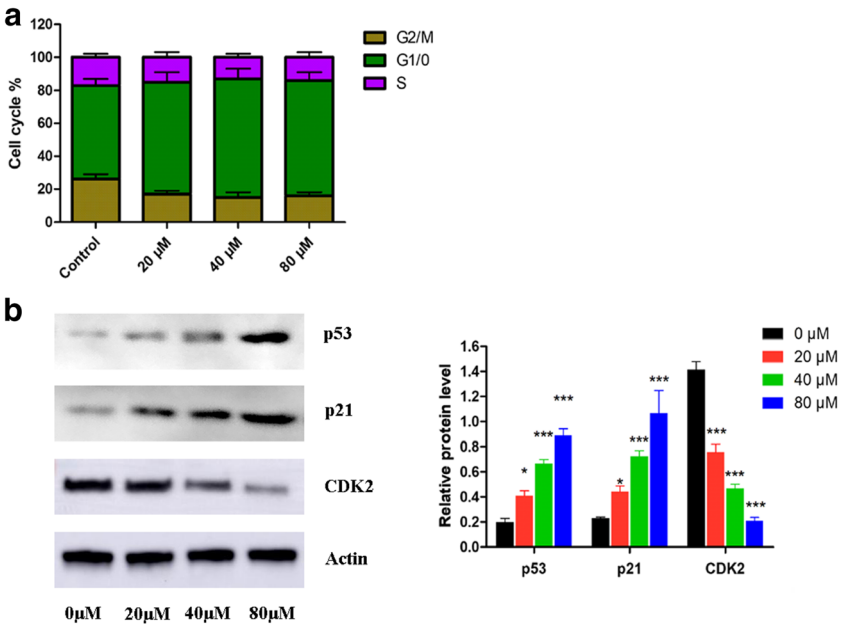

Fig. 5 **a** CPZ1344 increases the rate of G1-phase U87 cells detected by flow cytometry. **b** Quantification of cell cycle associated proteins normalized to β-actin expression to indicate GBM cell cycle status in response to CPZ1344 treatment. *$p < 0.05$, ***$p < 0.001$ vs. controls

effects of CPZ1344 on HUVEC vasculature were not mediated by its cytotoxic activity.

Discussion

CXCR4 is a seven-transmembrane GPCR, which has attracted attention as a co-receptor for the infection of HIV-1 [16]. Recent studies suggest that CXCR4 is not only a co-receptor for HIV, but plays a key role in cancer metastasis and neovascularization. Intense interest has emerged in blocking the ability of CXCR4 to interact with its ligands as an anti-cancer strategy. Short peptide CXCR4 antagonists have been reported, but the disclosure of small-molecular CXCR4 antagonists are limited [11]. AMD3100 is the first reported CXCR4 antagonist, which contains a bicyclam scaffold. Unfortunately, the application of AMD3100 in clinical was extraordinarily difficult due to its cardiotoxicity and lack of oral bioavailability. Developing novel CXCR4 antagonist based on AMD3100 and previously reported structure-activity relationship is a meaningful work [17]. In this study, CZP1344 was developed from WZ811 as a lead compound, by replacing the N-containing basic macrocyclic centers of AMD3100 to pyridine group. The evaluation of CPZ1344 demonstrated its ability to significantly inhibits the viability and migration of

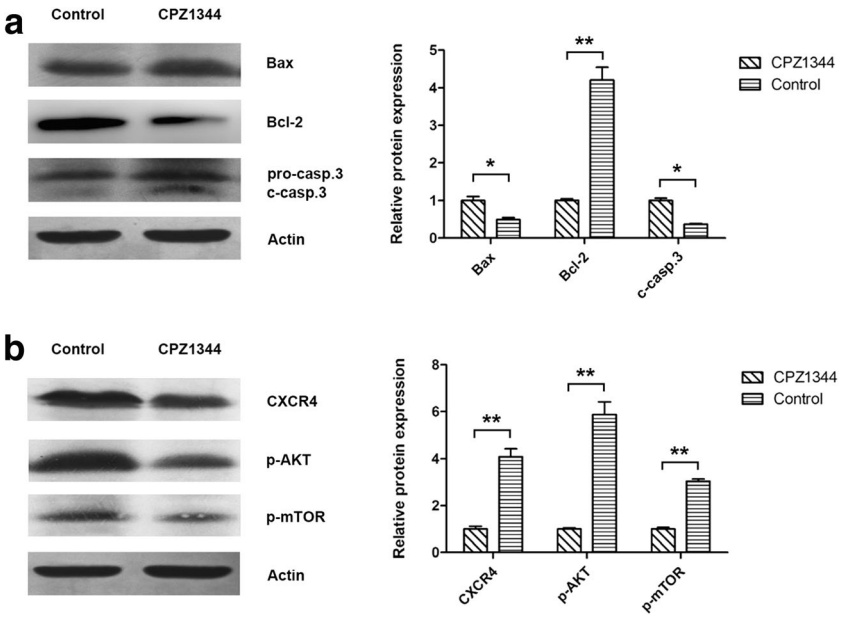

Fig. 6 **a** Bax, bcl-2, and caspase 3 expression were assessed by western blotting. Protein expression was quantified and normalized to β-actin expression to indicate GBM cell cycle status in response to CPZ1344 treatment. **b** CPZ1344 suppresses CXCR4 mediated AKT and mTOR induction as assessed by western blot analysis

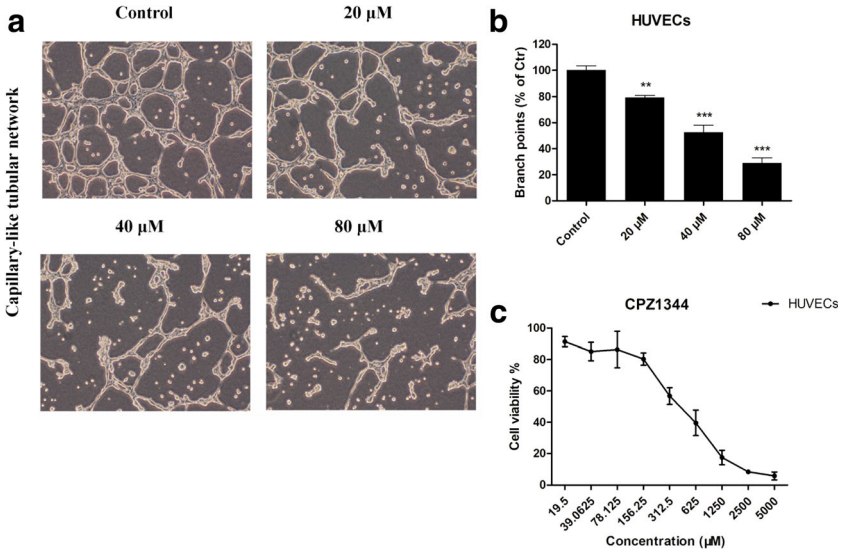

Fig. 7 Effects of CPZ1344 on the angiogenesis and viability of HUVECS. **a** The representative image clearly shows the formation of tubular structures in the HUVECs following treatment with a range of concentrations of CPZ1344 for 6 h. **b** Quantification of vascular branching. Branch point (sites of intersection of at least three tubes) number in each well was counted and expressed as a percentage of branch points formed in the control sample, taken as 100%, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. controls. **c** The cell rate viability assayed by MTT assay

cultured GBM cells, which warrants further exploration as an antitumor drug candidate.

Despite the tremendous advances in cancer therapeutics, the current standard treatment of GBM is still using maximal surgical tumor removal followed by combination of chemo-radiotherapy and temozolomide administration. Novel therapeutics with improved activity against GBM cells and tumors are urgently required. The CXCR4/CXCL12 axis is a key mediator of tumor development, including GBM, breast cancer and lung cancer, amongst others. The overexpression of CXCR4 positively correlates with a poor patient prognosis in metastatic cancer. Targeting CXCR4 with small molecules provided a promising strategy to interfere with glioma cell proliferation, survival and migration. Treatment with the CXCR4 antagonist AMD3100 alone or in combination with chemotherapeutic agents could induce GBM cell apoptosis and inhibit cell proliferation through the antagonism of CXCR4 [18]. In this study, we first confirmed the over expression of CXCR4 in both protein and mRNA levels in glioma cells. CXCR4 inhibitor CPZ1344, was then demonstrated to inhibit GBM proliferation and induce apoptosis without apparent toxicity. In addition, CPZ1344 induced G1 arrest in GBM cell lines. Taken together, these data highlight CPZ1344 as an attractive candidate for future anti-GBM therapeutics. However, it should be noted that due to the divergence between patient-derived glioma cells and GBM cells, further evaluations of CPZ1344 on patient-derived glioma cells are still need to prove its potency as an anti-GBM agent.

Inhibiting angiogenesis might be an effective strategy to prevent the growth, invasion and migration of GBM. CXCL12/CXCR4 are chemokine receptors that play key roles in the proliferative capacity of cancer cells. In this study, we also demonstrated that CPZ1344 exhibited anti-vascular activity, suggesting that its inhibition on the proliferation of GBM may be associated with inhibiting angiogenesis. VEGF is another validated target for GBM treatment, however, tumor relapse after VEGF antagonism treatment is a major concern. The combination of VEGF antagonism and chemotherapy became a trend in clinical application of VEGF antagonism to overcome tumor relapse [19]. The combination of CXCR4 and VEGF antagonism in clinical studies provides the rationale of combining CPZ1344 with VEGF inhibitors, such as bevacizumab in future studies to improve its efficacy and reduce tumor recurrence [20, 21].

Collectively, this study shows that CPZ1344 downregulates the expression of CXCR4 in GBM, leading to a loss of GBM cell migration, growth and angiogenesis. These studies further support the promise of CPZ1344 as a much-needed and novel anti-GBM agent.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

Ethical Approval All procedures performed in this study were in accordance with the ethical standards of the Affiliated Kunshan Hospital of Jiangsu University Ethics Committee for Scientific Research (No. 201901276) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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